(54) Title: RAPID ASSAY OF ACTIVATORS AND INHIBITORS OF CLOTTING

(57) Abstract

Assays and reagents are provided for the measurement of components that are involved in either enzyme-catalyzed degradation of a substrate, such as in fibrinogenolysis or fibrinolysis; or enzyme-catalyzed polymerization of a substrate, such as in fibrinogen polymerization into fibrin. The method involves using a fluorescent-labeled substrate in an enzyme-catalyzed reaction, and measurement of the component's effects on the fluorescent emission of the enzyme-catalyzed reaction such as quenching or dequenching, as a measure of the component's activity.

RAPID ASSAY OF ACTIVATORS AND INHIBITORS OF CLOTTING

FIELD OF THE INVENTION

The present invention relates to rapid and sensitive fluorescence assays and assay reagents for measuring body fluid components involved in protein
5   polymer formation and protein polymer degradation. More particularly, the invention discloses assays and assay reagents for the activity of blood components which are involved or active in clot formation, such as in fibrinogen polymerization; and for the activity of
10 components which are involved or active in clot degradation such as in fibrinogenolysis, or fibrinolysis.

BACKGROUND OF THE INVENTION

15 In the field of biomedicine, there is a significant need for bioassays useful to determine the activity of body fluid components, particularly enzymes such as proteases, on their respective target substrates. Although chromogenic or fluorogenic substrates have been
20 used in the past to assay the activity of enzymes, there is a need for a method or assay which is rapid, efficient, inexpensive, and highly sensitive.

For example, an illustration of the usefulness of such assays is the measurement of components involved in
25 fibrinogen polymerization, fibrinolysis, or fibrinogenolysis. Fibrinogen is the key structural protein in blood clot formation. It is a fibrous protein having three pairs of polypeptide chains held together by disulfide bonds. In fibrinogen polymerization , as
30 illustrated in Figure 1A, fibrinogen is a substrate for thrombin proteolytic activity. Thrombin catalyzes the

release of small peptides, fibrinopeptides A and B, from
the chains of fibrinogen. The removal of the
fibrinopeptides from the fibrinogen substrate results in
molecules which polymerize spontaneously into fibers in
5   forming fibrin. After serving its role as the protein
matrix of a blood clot, and in tissue repair, fibrin is
then removed or degraded in the process of fibrinolysis.
        As illustrated in Figure 1B, the inactive
precursor, plasminogen, is converted into the active
10   proteolytic enzyme, plasmin. In fibrinolysis, plasmin
catalyzes the fibrin substrate into soluble degradation
products. Further, since plasmin is enzymatically
active against fibrinogen, fibrinogenolysis is a process
by which fibrinogen substrate is degraded into soluble
15   products.
        Measurement of the various enzymes, inhibitors,
activators, and protein substrates involved in clot
formation, inhibition of clot formation, or dissolution
of clots (thrombolysis) has medical applications. For
20   example, thrombosis is an important cause of human
illness and death. Thrombolytic agents are increasingly
used in pharmacological prevention and/or dissolution of
formed thrombi such as in acute myocardial infarction.
        Various methods for assaying various blood factors
25   involved with the formation, inhibition, or degradation
of clots have been described previously. Typically,
these bioassays involve chromogenic assay techniques
requiring enzyme labelling of the substrate, addition of
the component(s) for which activity is to be measured,
30   and subsequent detection of the amount of color formed.
Similar bioassays have been described without the use of
chromogens, but instead measure turbidity once the
reaction has taken place. Disadvantages of these types
of assays, particularly for the measurement of
35   components which are normally in low concentrations in
body fluid, include: susceptibility to diffusion

artifacts; relative time consumption to perform the
assay and obtain the results; the concentration of
substrate that is required for the assay; lack of
kinetic information of the process being measured; and
5   lack of sensitivity of detection to the picomolar
nanomolar level. Thus, for applications such as
monitoring thrombolytic agent therapy in a clinical
setting, previously described methods have been found to
be time-consuming and insufficiently sensitive.
10   Therefore, there is interest and need in providing
assays which are rapid, highly sensitive, provide
measurements of body fluid components which reliably
reflect in vivo activity, and which can be used to
detect and quantitate a wide variety of analytes
15   including enzymes, enzyme inhibitors, and enzyme
activators.

SUMMARY OF THE INVENTION

A novel method is provided for detecting and
20   quantitating body fluid components involved in either
enzyme-catalyzed protein polymer formation or enzyme-
catalyzed protein polymer degradation. The method
involves tagging a substrate with a fluorescent label.
The substrate is the protein template with which
25   molecular assembly with the respective enzyme occurs,
and is also the molecule, in a modified form, that
generates a signal that is directly detected since it
has incorporated therein, molecules of fluorescent
label. Depending on whether the enzymatic reaction
30   involves polymerization or degradation, quenching or
dequenching of the fluorescence emission is measured
which relates directly to the kinetics and quantitation
of modification of the substrate. Therefore, with a
known amount of substrate, the activity of the
35   respective enzyme is directly detected. The
fluorescence emission also provides an indirect means

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for the quantitation of activity of an enzyme inhibitor
or activator. The method avoids diffusion-limitation
artifacts, and is useful for immediate (within minutes)
determination of a component's activity.

5   BRIEF DESCRIPTION OF THE DRAWINGS
       FIG. 1A is a schematic representation of fibrinogen
       polymerization.
       FIG. 1B is a schematic representation of fibrinolysis
10   and fibrinogenolysis.
       FIG. 2A is a graph illustrating dependence of
       fluorescence intensity of 30 nM fluorescent-labeled
       fibrinogen upon addition of 0.1 to 1.0 U/ml thrombin at
       0.1 M NaCl.
15   FIG. 2B is a graph illustrating dependence of
       fluorescence intensity of 30 nM fluorescent-labeled
       fibrinogen upon addition of 0.1 to 1.0 U/ml thrombin at
       0.3 M NaCl.
       FIG. 3A is a graph showing the effects of addition of
20   unlabeled fibrinogen to 35 nM of fluorescent-labeled
       fibrinogen prior to addition of 0.5 U/ml thrombin in 0.1
       M NaCl.
       FIG. 3B is a graph showing the effects of addition of
       unlabeled fibrinogen to 35 nM of fluorescent-labeled
25   fibrinogen prior to addition of 0.5 U/ml thrombin in 0.3
       M NaCl.
       FIG. 4 is a graph showing fluorescence intensity of 250
       nM fluorescent-labeled fibrinogen and 750 nM unlabeled
       fibrinogen upon addition of 10 nM plasmin.
30   FIG. 5 is a graph represents a reciprocal plot showing
       the effect of fluorescent-labeled fibrinogen
       concentration (1.25 to 20 nM) on plasmin-mediated
       fibrinogenolysis. The theoretical curve using Equations
       5 and 6 is shown for $k_{cat} = 0.479$ sec^{-1} and $K_d = 0.42$ μM.
35   FIG. 6 is a graph showing the effect of increasing
       plasmin concentration on the initial rate of fragment X

generation at constant concentration of fibrinogen (10.0 nM). The theoretical curve using Equations 5 and 6 is shown for $k_{cat} = 0.479$ sec^{-1} and $K_d = 0.42$ μM.

    FIG. 7 is a graph showing the effect of dequenching by
5   addition of ϵ-amino caproic acid-plasmin complex to fluorescent-labeled fibrinogen on dequenching compared to an equal amount of plasmin (1.0 nM) added to fluorescent-labeled fibrinogen.

    FIG. 8 is a graph showing the effect on dequenching by
10   addition of ϵ-amino caproic acid (0.1 to 10 nM) to fluorescent-labeled fibrinogen (20 nM) undergoing degradation by plasmin (1.0 nM).

    FIG. 9 is a graph showing the effect of fibrin concentration on the initial rate of dequenching by
15   plasmin (5.0 nM) using coarse fibrin fibers (formed in 0.1 M NaCl) or fine fibrin fibers (formed in 0.3 M NaCl).

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

20   The method according to the invention for detecting and quantitating body fluid components involved in either enzyme-catalyzed protein polymer formation or enzyme-catalyzed protein polymer degradation is performed by using a substrate having been tagged with a
25   fluorescent label. Because the substrate is the protein template with which molecular assembly with the respective enzyme occurs, and is also the molecule which in a modified form is directly detected since it has incorporated therein molecules of fluorescent label,
30   enzymes that are dilute in solution achieve very high concentrations locally when assembled on the substrate. This gives the fluorescent-labeled substrate a very sensitive signal when used in low concentrations (e.g., 1 to 10 nM). This concentration is about 1000 fold
35   lower than typical μM levels for fluorogenic or

chromogenic substrates used in other assays that have no
ability to concentrate molecules locally on a template.

In measuring components involved in a pathway
which leads to polymerization of the substrate, the
5   fluorescence of the label molecules of the polymerized
substrate is quenched (reduced fluorescence emission),
relative to the basal level of fluorescence prior to
polymerization. The fluorescence quenching is due to
enhanced neighbor-neighbor interactions between the
10 fluorescent label molecules in the polymerized
substrate. Measurement of quenching during the
polymerization to an end point determination provides
for information on the kinetics of the reaction.
Measurement of fluorescence emission at the end point of
15 quenching, relative to the basal level of fluorescence
emission, can be used to quantitate enzyme activity, or
enzyme activators or inhibitors, depending on the
components included in the reaction mixture.

In measuring components involved in a pathway
20 which leads to degradation or dissolution of the
substrate or polymerized form of substrate, the
fluorescence of the label molecules of the substrate or
polymerized form of substrate is dequenched (increased
fluorescence emission), relative to the basal level of
25 fluorescence prior to degradation or dissolution. The
fluorescence dequenching is due to an increase in the
distance between neighboring molecules of fluorescent
label as a result or unfolding or cleavage of the
substrate or polymerized from of substrate; i.e.,
30 thereby being free from proximity-based quenching.
Measurement of dequenching during degradation or
dissolution to an end point determination provides for
information on the kinetics of the reaction.
Measurement of fluorescence emission at the end point of
35 dequenching, relative to the basal level of fluorescence
emission, can be used to quantitate enzyme activity, or

enzyme activators or inhibitors, depending on the
components included in the reaction mixture.

Body fluid components or components are terms used
herein to refer to enzymes, enzyme inhibitors or enzyme
5   activators found in body fluid which are involved in
either a pathway of protein substrate polymerization, or
a pathway of protein substrate dissolution or
degradation. Enzyme inhibitors are proteins or factors
which have activity that directly or indirectly inhibits
10 or inactivates an enzyme. Although not found naturally
occurring in body fluid, a pharmaceutical having enzyme
inhibitor activity, for the purpose of the present
invention, will also be included in the term "enzyme
inhibitor". Enzyme activators are proteins or factors
15 which interact with an enzyme in forming an active
enzyme or active enzyme complex. Although not found
naturally occurring in body fluid, a pharmaceutical
having enzyme activator activity, for the purpose of the
present invention, will also be included in the term
20 "enzyme activators". Analytes are substances, including
the protein substrate or other body fluid components
that are detected and quantitated by the method of the
present invention. "Measurement directly" is used
herein to mean that the fluorescence emission changes
25 reflect the direct effect an enzyme has on the
substrate. "Measurement indirectly" is used herein to
mean an inhibitive, competitive, enhancing secondary
interaction on the direct effect between enzyme and
substrate. "Basal level of fluorescence emission" is
30 used herein to mean a the fluorescence emission from a
reference reaction. For example, when an enzyme's
activity is being measured, the basal level of
fluorescence would be that from a reaction containing
the substrate and reaction mixture alone. When the
35 activity of an enzyme inhibitor or enzyme activator is
being measured, the basal level of fluorescence would be

that from a reaction containing the substrate, enzyme, and reaction mixture.

Various protocols may be employed for the method according to the present invention depending upon the

5   substrate used, and the different analytes to be measured. Similarly, different reagents may be necessary, and various components may need be added, depending upon the substrate used and analyte(s) to be measured. However, common to all embodiments of the

10   method of the present invention is that the substrate in the method is a dimeric or oligomeric molecule, preferably naturally occurring as a body fluid component, which a) is labeled with fluorescent molecules; b) is modifiable in either an enzyme-

15   catalyzed polymerization reaction or an enzyme-catalyzed degradation or dissolution reaction; c) where upon modification of the fluorescent-labeled substrate, a change in fluorescence emission results; and d) measurement of the difference between the fluorescence

20   emission as a result of the reaction and the basal level of fluorescence emission (prior to substrate modification) directly relates to substrate concentration and/or enzyme activity and indirectly relates to the presence of absence of other components

25   involved in that pathway. Thus, the substrate is a template with which molecular assemblies that occur in the body are recreated in the assay. The substrate being a dimer or oligomer facilitates the achievement of fluorescent labelling that is internally quenched.

30   Further common to all embodiments of the present invention, the various reagents or components of a reaction mixture are mixed in solution, and then incubated for a sufficient time to allow for a reaction to occur. Thus, in another aspect the method of the

35   present invention differs from methods using chromogenic or fluorogenic substrates bound to a solid support, by

obviating the need for a wash phase in which the solid
phase is washed to remove unbound or non-specifically
bound label. It is important to note that the method
according to the present invention is capable of
5   detecting, and measuring in both a kinetic (if desired)
and endpoint fashion, the direct or indirect action of
an unknown amount of analyte on the fluorescent-labeled
substrate.

In one particular embodiment of the present
10   invention, one or more components required in the
processes of blood coagulation or blood fibrinolysis is
the analyte. Since fibrinogen and/or fibrin are the key
reactive species in these processes, and since there are
multiple components that affect the processes, numerous
15   embodiments of assays using fluorescent-labeled fibrin
or fibrinogen may be performed according to the method
of the present invention. Further, in addition to blood
components being tested as analytes in the method
according to the present invention, synthetic materials
20   such as pharmaceuticals which may activate or inhibit a
blood component involved in the processes of blood
coagulation or blood fibrinolysis, may be the analyte.
In using either fibrin or fibrinogen as the substrate,
depending on the analyte to be measured, the method of
25   the present invention is an assay reflective of both
binding and activity. For example, according to the
method of the present invention, plasmin must bind the
fluorescent substrate in order for a signal change from
the basal level of fluorescence to occur; i.e., to
30   initiate detection of plasmin enzyme activity. Thus,
the assay of the present invention can detect either the
activity of components (e.g. ϵ-amino caproic acid) which
inhibit the binding of the enzyme (plasmin), or the
activity of components that affect or inhibit the active
35   site (e.g., α_2- Antiplasmin) of the enzyme (plasmin). In
contrast, assays in the art which contain a peptide of

fibrin or fibrinogen as a substrate typically measure
only components that affect plasmin activity, but not
plasmin binding. Therefore the substrate used with the
present invention is a natural or natural-like (e.g.,
5   recombinant) substrate which more accurately represents
the particular pathway in body fluids compared to the
smaller synthesized (e.g. peptide) substrates.
        This embodiment of the assay is particularly useful
for the detection and quantitation of fibrinolytic
10 components with accuracy, rapidity, and sensitivity
(nanomolar to picomolar range) not achievable in current
commercially available assays. The accuracy, rapidity
and sensitivity provided by the method of the present
invention will particularly be appreciated in a clinical
15 setting in which thrombolytic therapy is being
monitored. Further, the assay of the present invention
utilizes fluorescent labeling, and thus avoids the use
radioactive labels and the attendant problems with their
use. Therefore in a clinical setting, the assay may be
20 carried out simply in a cuvette or a 96-well plate and
then measured using a fluorimeter.
        For example, when a enzyme that is active in clot
formation or clot degradation is the analyte, it will be
necessary to only include a substrate, such as
25 fluorescent-labeled fibrinogen or fibrin depending on
the enzyme, in a reaction mixture in solution such as
diluted plasma. Thus, a blood sample would be taken;
plasma from the blood sample would be isolated, diluted,
and then added to plasma deficient in the enzyme to be
30 tested but containing a known amount of fluorescent-
labeled substrate. Thus, an enzyme-catalyzed reaction
of the substrate is a measurable effect of the activity
of the enzyme contained in the sample.
        If an activator or inhibitor is the analyte, then
35 components (i.e. precursors to the enzyme, and/or
enzyme) other than the analyte required in that

particular pathway may need be added if they are not already contained within the reaction mixture or plasma, in addition to the fluorescent-labeled substrate. The measurable effect would then relate to the activity of
5   the analyte in the sample. Knowing the concentration of the enzyme and fluorescent-labeled substrate in a reaction will allow for indirect quantitation of the activity of an inhibitor or activator present in the sample.

10   The following examples are offered for the purposes of illustration, and not by way of limitation.

EXAMPLE 1

        Various fluorescent molecules are known in the art
15   which are suitable for use to label a protein substrate for the method of the present invention. Because substrates for the method of he present invention are dimeric or oligomeric molecules, incorporation of fluorescent label into more than one chain of the
20   substrate will lead to quenching due to molecular proximity. A partial, but representative list of the class of such fluorescent molecules is illustrated as Table 1, and includes the respective excitation wavelength (EX) and emission wavelength (EM) for
25   detection (in nanometers, "nm"). Common fluorescent labels for protein labeling include amine-reactive probes which are reactive to end terminal amines and the lysines of the protein substrate; isothiocyanates; succinimidyl esters; sulfonyl chlorides which are
30   conjugated to the substrate through amine residues and tyrosine; sulfhydryl-reactive probes that react with thiols found in proteins; and the like. Depending on the fluorescent molecule used, methods of incorporating or tagging the substrate with the fluorescent molecule
35   label include attachment by covalent or noncovalent means.

Table 1

Fluorescent molecule	EX	EM
Fluorescein isothiocyanate (FITC)	491 nm	515 nm
Tetramethylrhodamine isothio-cyanate (TRITC)	540 nm	567 nm
carboxytetramethylrhodamine succinimidyl ester	540 nm	567 nm
carboxyfluorescein succinimidyl ester	491 nm	515 nm
TEXAS RED^R sulfonyl chloride	589 nm	615 nm
7-diethylamino-3-((4'iodoacetyl)amino) phenyl0-4-methylcoumarin	382 nm	472 nm
5-iodoacetamidofluorescein	491 nm	515 nm
tetramethylrhodamine-5-(and 6)-iodoacetamide	540 nm	567 nm
N-(4,4-difluoro-5,7- dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-N'iodoacetyl-ethylenediamine	504 nm	510 nm
Coumarin malemide	385 nm	471 nm
Fluorescein malemide	490 nm	515 nm
Rhodamine malemide	542 nm	566 nm

The protocol for labeling of substrate with
fluorescent molecule may vary depending upon the
fluorescent molecule used. Such protocols are known in
the art for the respective fluorescent molecule. For
example, a general protocol for labeling the protein
substrate with FITC or TRITC is as follows.
The protein substrate, such as fibrinogen, in a
concentration from 0.1 to 10 mg/ml is dissolved in 0.1
to 0.2 M bicarbonate buffer (pH 8.3 to 9). The
substrate is then incubated with FITC or TRITC (1 mg/ml)
with continuous stirring for 1 hour at 22°C in a
labeling buffer of 0.1 M sodium bicarbonate (pH 9.0).
The reaction is stopped with hydroxylamine (0.15 M final

concentra- tion, freshly prepared), incubated for 1
hour, after which the labeled substrate is dialyzed
extensively in the dark at 4°C, and then stored at -75°C
until used (since only nanomolar levels of substrate are
5 required per reaction). Alternatively, to remove
unincorporated fluorescent label from the labeled
substrate, the substrate can be subjected to molecular
sieve chromatography.

Generally, the protocol for labeling substrate with
10 amine-reactive probes is the same as that of labelling
with FITC or TRITC except that the fluorescent label
(e.g. TEXAS RED^R sulfonyl chloride) is incubated with the
protein substrate for 1 hour at room temperature or 4°C
in 0.1 to 0.2 M sodium bicarbonate solution at pH 8.3.
15 The general protocol for labeling the substrate with a
fluorescent molecule comprising a haloacetyl derivative
(type of sulfhydryl-reactive label) is as follows. The
protein substrate, such as fibrinogen, in a
concentration of 0.1 to 10 mg/ml is incubated with
20 iodoacetamide reagent at 0.1 to 10 mg/ml concentration
for 1 hour at room temperature under neutral buffer
conditions (pH < 8.0) in the dark. The labeled protein
can be separated from unincorporated label using
molecular sieve chromatography or extensive dialysis in
25 the dark. The resultant fluorescent-labeled substrate
may then be stored frozen. For malemide derivatives
(type of sulfhydryl-reactive label) the protein
substrate, such as fibrinogen, in a concentration of 0.1
to 10 mg/ml is incubated with maleimide reagent at 0.1
30 to 10 mg/ml concentration for 1 hour at room temperature
under neutral buffer conditions (pH < 8.0) in the dark.
The labeled protein can be separated from unincorporated
label using molecular sieve chromatography or extensive
dialysis in the dark.

35

EXAMPLE 2

There are some general considerations in practicing the method of the present invention. The assay reaction is performed in the liquid phase contained within a
5   cuvette, the well of a microtiter plate, or like container suitable for fluorimetry. Typically, the inner surface of the container is preincubated with an inert protein, such as serum albumin, to substantially reduce or eliminate nonspecific binding of the
10 fluorescent-labeled substrate to the surface. Alternatively, transparent, low protein-binding materials may be used as incubation wells.

The fluorescent-labeled substrate is diluted in a reaction buffer comprising a physiological saline
15 solution. Likewise, if the sample to be analyzed requires dilution, the diluent should also comprise such a saline solution. A general saline buffer using HEPES, Tris, or phosphate buffer is suitable. Salt concentrations in the buffer can range from 0.1 M to 0.5
20 M salt such as NaCl. Thus, an exemplary buffer comprises 0.1 M NaCl, 5 mM $CaCl_2$, and 10 to 50 mM Tris-HCl (pH 7.4). In general, a zinc salt (zinc ion, Zn^{2+}) should be avoided as it may precipitate out of solution a substrate such as fibrin. Buffer components should
25 also be chosen to include those which do not inhibit polymerization or dissolution/degradation processes.

A fluorescence emission baseline is established before the sample containing the analyte is added to the reaction mixture. In that regard, the reaction should be
30 performed to avoid the presence of any contaminating strongly fluorescent or quenching materials that overlap with the excitation or emission wavelength of the fluorescent-labeled substrate. Since the sample containing the analyte to be measured may be a body
35 fluid, red blood cells may be present. The inclusion of hemoglobin or red blood cells in the reaction is not

recommended, but may be tolerated in some conditions.
The reaction buffer may also comprise platelet poor
plasma into which the labeled-substrate is added.

The reaction is initiated by the addition of
5   critical enzyme in the sample to the fluorescent
substrate (e.g., thrombin to fibrinogen). The reaction
mixture is then excited with light at a wavelength
dependent on the fluorescent molecule used to label the
substrate (see for example, Table 1). For example,
10 using FITC-fibrinogen in the method according to the
present invention, the optimal excitation is blue light
(488 nm). Fluorescent emission is then measured with a
light detector (i.e., photo-multiplier tube) in a
fluorimeter. For example, for FITC-labeled substrate,
15 green emission light (515 nm) is collected using a
photomultiplier tube inside a fluorimeter. The intensity
of emission of fluorescent light is monitored
continuously by sampling the emission at 0.5 to 10 times
per second. The development of signal is dependent on
20 the initial state of the fluorescent-labeled substrate
and reaction events occurring on the substrate
(resulting in either polymerization or degradation/
dissolution). However, a range of typical reaction
times to an end-point determination is 1 to 5 minutes.
25 The assay may be carried out at temperatures ranging
from about 4°C to about 37°C, and preferably a room
temperature or 37°C.

Other reagents (proteins, inhibitors, activators,
etc.) can be added to the fluorescent-labeled substrate
30 prior to start of the reaction. Alternately, it is
possible to preincubate components of the reaction
mixture together prior to addition of fluorescent-
labeled substrate to the incubation mixture. For
example, a preincubation reaction can include urokinase
35 incubated with plasminogen to generate plasmin. In
another embodiment, tPA (tissue plasminogen activator)

can be incubated with unlabeled fibrin in the presence
of plasminogen to generate plasmin. In either example,
the preincubation reaction mixture can then be added to
fluorescent-labeled fibrin or fluorescent-labeled
5   fibrinogen substrate.

The use of a fluorescent-labeled substrate, as
defined herein, to assay enzyme activities in a standard
fluorimeter is novel. Because the substrate is a
dimeric or oligomeric molecule, preferably naturally
10   occurring as a body fluid component, and on which
molecular assemblies that occur in the body are
recreated, the molecular size of the substrate to be
used in the method of the present invention is greater
than about 100,000 daltons (e.g. the molecular size of
15   fibrinogen is 340,000 daltons). Thus, the method of the
present invention of following the fluorescence signal
of such substrate during polymerization (with quenching
of the signal) or proteolysis (with dequenching of the
signal) is novel and very distinct from fluorescence
20   polarization anisotropy measurements which are not
useful for substrates having a molecular size above
approximately 65,000 daltons.

Concerning assay reagents comprising components,
other than the analyte, to be added to facilitate
25   process of modification of the fluorescent-labeled
substrate, in many instances it will not be necessary to
use such components from the same species as the species
of the analyte. For example, it is known that non-human
components involved in fibrinolysis display cross-
30   activity with the respective components found in
humans. Thus, while the method of the present invention
is preferably performed to measure analytes involved in
human processes, assay reagents may include components
from other species, if desirable. Further, various
35   assay reagents useful for the particular process to be
measured, can be included in a kit form to facilitate

practice of the method of the present invention. In this regard, fluorescent-labeled substrate (e.g., FITC-fibrinogen) is stable for at least 9 months, and may be stable up to two years, with proper storage conditions.

5

EXAMPLE 3

Fibrinogen Polymerization

An assay for fibrinogen polymerization according to the method of the present invention was performed in accordance with materials and methods outlined in

10 Examples 1 and 2. Fibrinogen was labeled with FITC using a 0.1 M sodium bicarbonate buffer. After stopping the reaction with hydroxylamine (0.15 M final concentration), the FITC-fibrinogen was dialyzed extensively before storage. The buffer for the

15 polymerization reaction comprised either 0.1 M NaCl, 0.05 M Tris-HCl, 5mM CaCl₂ ("buffer 1") or 0.3 M NaCl, 0.05 M Tris-HCl, 5mM CaCl₂ ("buffer 2"). Cuvettes were incubated with 10μM bovine serum albumin for 30 minutes

20 to minimize adsorption of substrate to the cuvette walls.

In a series of reactions to produce fibrinogen polymerization into fibrin fibers, thrombin (final concentrations ranging from 0.1 U/ml, 0.25 U/ml, 0.5

25 U/ml and 1 U/ml) was mixed for about 5 seconds in either buffer 1 or buffer 2 containing a dilute FITC-fibrinogen (<40 nM) solution. Polymerization was monitored by exciting the reaction mixture with blue light (488 nm) and monitoring the fluorescence emission at 515 nm using

30 a fluorimeter. The addition of thrombin to FITC-fibrinogen led to rapid and dose-dependent reduction of fluorescence emission due to quenching. At these extremely low concentration of fluorescent-labeled substrate (< 40 nM), the solutions were optically clear

35 and the drop in fluorescence was not due to turbidity or scattering effects during polymerization. Release of

fibrinopeptides A and B from fibrinogen, mediated by
thrombin action, caused no detectable dequenching of the
FITC moieties on fibrin monomers.

The initial rate of quenching was dependent on the
5   initial concentration of thrombin, as illustrated in
Fig. 2A (30 mM FITC-fibrinogen polymerized in buffer 1)
and in Fig. 2B (30 mM FITC-fibrinogen polymerized in
buffer 2). At the lowest concentrations of thrombin
used, a comparatively significant lag time was observed
10 before dequenching proceeded. After generation of
sufficient monomer and protofibril extension, lateral
aggregation of fibrils proceeded. Once dequenching
began, the maximum rate of dequenching with time
appeared to be largely independent of thrombin
15 concentration, as seen by the similar maximum slopes on
curves in Figs. 2A or 2B. Also, the final extent of
quenching appeared to be modulated by the thickness of
the fiber. At low thrombin concentration and low salt
(buffer 1) the thicker fibers that formed (taking longer
20 to form) resulted in a greater final extent of quenching
compared to the thinner fibers formed at higher thrombin
concentrations (Fig. 2A). In high salt, favoring the
formation of thinner fibers, the modulation of the final
extent of quenching by adjusting the thrombin
25 concentration was qualitatively similar to reactions
with low salt, but the final differences were much
smaller. As seen in Figs. 2A and 2B, at all thrombin
concentrations, the lag phase was considerably longer in
buffer 2 as compared to in buffer 1, suggesting the
30 importance of lateral aggregation during dequenching
(i.e., protofibril extension is fast at higher salt
concentrations). However, when quenching achieved its
maximal rate at a given stage of the polymerization,
there was little difference between that rate for low
35 (buffer 1) and high (buffer 2) salt concentrations.

Addition of unlabeled fibrinogen to the reaction mixture attenuated the thrombin-induced quenching in a dose-dependent manner (Figs. 3A and 3B). This result would not be seen for a scattering-based mechanism of
5 loss of fluorescence signal. Increasing the concentration of unlabeled fibrinogen reduces the probability of two FITC-fibrin monomers either being incorporated sequentially in a protofibril or interacting within a given cross-section of fiber.
10 Increasing the unlabeled fibrinogen in the reaction altered the initial quenching rate slightly (at constant thrombin concentration) and reduced the final extent of quenching in a dose-dependent manner. At a 5:1 ratio of unlabeled fibrinogen to FITC-fibrinogen, the quenching
15 during polymerization was completely eliminated in fine fibers (Fig. 3A) while a small amount of quenching occurred at this ratio during polymerization of coarse fibers (Fig. 3B). This is consistent with an increased probability of two or more labeled FITC-fibrin monomers
20 quenching each other in a thick fiber cross-section as compared to the quenching probability in a thin fiber-cross section which contains less fibrils. Thus, the quenching during polymerization is due to a mechanism involving molecular proximity which is achieved by two
25 processes: fibril extension and fibril aggregation.
It was also observed that polymerization of FITC-fibrinogen by 1 U/ml of thrombin in the presence 0.1, 10, and 100 μM Gly-Pro-Arg-Pro ("GPRP", a peptide that prevents polymerization; Laudano et al., 1980 *Biochem.*
30 19:1013-1019) reduced in a dose-dependent manner the rate and extent of quenching associated with polymerization. Over 80% of the thrombin-induced quenching was prevented when 100 μM GPRP was added before polymerization. Similar to the findings given
35 for unlabeled fibrinogen, this finding with GPRP

- 20 -

verifies and validates the novel mechanism by which the
assay works.

EXAMPLE 4
Thrombin Activity Assay

5   An assay for thrombin activity in fibrinogen
polymerization according to the method of the present
invention can be performed in accordance with materials
and methods outlined in Examples 1, 2, and 3. The
amount of biological sample, comprising body fluid to be
10   tested for thrombin activity, to be added to the
reaction mixture can range from about 1 μl to 100 μl,
depending on the body fluid and if dilution of the
sample is desirable. Likewise, one skilled in the art
will appreciate that the amount of reaction buffer to
15   which the sample is added (e.g., 0.1 ml to 2.5 ml) and
the amount of FITC-fibrinogen in the reaction (e.g., 1
nM to 50 nM) will depend on the sample to be analyzed,
and the mode (cuvette, microtiter well, etc.) in which
20   the reaction takes place.
     The sample is added to the reaction buffer
containing FITC-fibrinogen. Polymerization is then
monitored by exciting the reaction mixture with blue
light (488 nm) and monitoring the fluorescence emission
25   at 515 nm using a fluorimeter. In a parallel set of
reactions, one or more thrombin standards (e.g., between
0.1 U/ml and 10 U/ml) may be used to calibrate the assay
of the sample. The extent of quenching at the end of
desired time interval (e.g., 5-25 minutes) is related to
30   the thrombin activity, and hence the thrombin
concentration, present in the sample. Since plasmin, if
present in the sample, may cause fibrinogenolysis in an
assay intended to measure thrombin activity in
fibrinogen polymerization, a plasmin inhibitor may be
35   added when measuring thrombin activity. For example,
added to the reaction buffer containing the thrombin and

FITC-fibrinogen may be a plasmin inhibitor, such as ε-
amino caproic acid, to eliminate interference by plasmin
contained in the sample.

5   If desired, the specificity of the reaction for
thrombin activity may be validated by the addition of
specific thrombin inhibitors, into the reaction mixture
containing the sample and FITC-fibrinogen. Such
thrombin inhibitors include natural inhibitors like
antithrombin III, and synthetic inhibitors like

10   benzamidine and D-phenylalanyl-L-prolyl-L-arginine
chloromethyl ketone. Addition of a known amount of
thrombin inhibitor will increase the amount of sample
required (i.e., increase the amount of thrombin) to be
added to obtain equivalent fluorescence emission. From

15   the percent inhibition and from the thrombin
concentration in the absence of inhibitor, can be
calculated the increased thrombin activity
(concentration) required to obtain equivalent activity.

                              EXAMPLE 5
20   Heparin Activity Assay (reverse thrombin assay)
          Heparin and its variants are used in
anticoagulation therapy, wherein it prevents fibrinogen
polymerization by accelerating complexation of thrombin

25   with antithrombin. An assay for heparin activity in
fibrinogen polymerization according to the method of the
present invention can be performed in accordance with
materials and methods outlined in Examples 1, 2, 3, and
4.

30   The sample containing an unknown amount of heparin
activity is added to the reaction buffer simultaneously
with a known amount of thrombin (e.g., 1 U/ml),
antithrombin (e.g., 0 to 5000 U/ml), and containing
FITC-fibrinogen (e.g., 10 nM). Polymerization is then

35   monitored by exciting the reaction mixture with blue
light (488 nm) and monitoring the fluorescence emission

at 515 nm using a fluorimeter. A parallel set of
standard reactions containing thrombin, antithrombin,
FITC-fibrinogen may be used to determine the maximum
amount of fluorescence emission (quenching) during the
5   desired time interval. The extent of interference of
fibrinogen polymerization (i.e., inhibition of
quenching) at the end of desired time interval in the
reaction containing the sample, is related to the
heparin activity, and hence the heparin concentration,
10   present in the sample. Thus, from the percent
inhibition of fibrinogen polymerization (as observed by
a reduction in quenching) as compared to the
standard(s), can be calculated the heparin activity
(concentration) in the sample. Further, if desired, a
15   parallel set of standard reactions may be performed
containing thrombin, antithrombin, a known concentration
of heparin, and FITC-fibrinogen for use to calculate the
heparin activity (concentration) in the sample.

EXAMPLE 6

20   **Antithrombin Activity Assay (reverse thrombin assay)**
Antithrombin is a component that may be found in
body fluid, wherein it prevents fibrinogen
polymerization by direct suicide complexation with
25   thrombin. An assay for antithrombin activity in
fibrinogen polymerization according to the method of the
present invention can be performed in accordance with
materials and methods outlined in Examples 1, 2, 3, and
4.
30   The sample containing an unknown amount of
antithrombin activity is added to the reaction buffer
simultaneously with a known amount of thrombin (e.g., 1
U/ml), heparin (e.g., 0 to 5000 U/ml), and containing
FITC-fibrinogen (e.g., 10 nM). Polymerization is then
35   monitored by exciting the reaction mixture with blue
light (488 nm) and monitoring the fluorescence emission

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at 515 nm using a fluorimeter. A parallel set of
standard reactions containing thrombin, antithrombin,
FITC-fibrinogen may be used to determine the maximum
amount of fluorescence emission (quenching) during the
5   desired time interval. The extent of interference of
fibrinogen polymerization (i.e., inhibition of
quenching) at the end of desired time interval in the
reaction containing the sample, is related to the
antithrombin activity, and hence the antithrombin
10   concentration, present in the sample. Thus, from the
percent inhibition of fibrinogen polymerization (as
observed by a reduction in quenching) as compared to the
standard(s), can be calculated the antithrombin activity
(concentration) in the sample. Further, if desired, a
15   parallel set of standard reactions may be performed
containing thrombin, heparin, a known concentration of
antithrombin, and FITC-fibrinogen for use to calculate
the antithrombin activity (concentration) in the sample.

EXAMPLE 7
20
Fibrinogenolysis
An assay for fibrinogenolysis according to the
method of the present invention was performed in
accordance with materials and methods outlined in
25   Examples 1 and 2. Fibrinogen was labeled with FITC
using a 0.1 M sodium bicarbonate buffer. After stopping
the reaction with hydroxylamine (0.15 M final
concentration), the FITC-fibrinogen was dialyzed
extensively before storage. The buffer for the lysis
30   reaction comprised either 0.1 M NaCl, 0.05 M Tris-HCl,
5mM CaCl$_2$ ("buffer 1") or 0.3 M NaCl, 0.05 M Tris-HCl,
5mM CaCl$_2$ ("buffer 2"). Cuvettes were incubated with
10μM bovine serum albumin for 30 minutes to minimize
adsorption of substrate to the cuvette walls.
35   To illustrate plasmin-mediated fibrinogenolysis
into degradation products, 10 nM of plasmin was mixed

for about 5 seconds in the reaction buffer containing 250 nM FITC-fibrinogen. Substrate degradation was monitored by exciting the reaction mixture with blue light (488 nm) and then simultaneously monitoring the
5   fluorescence emission at 515 nm using a fluorimeter. The action of plasmin on FITC-fibrinogen produced an immediate large increase in fluorescence intensity (dequenching) in a plasmin-dependent manner (FIG. 4). The detection limit has been found under routine
10 conditions to be at least 10 picomolar plasmin.
        Degradation events were also monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as indicated by the arrows representing sampling times (FIG. 4). Four fragments (X, Y, D and E) were
15 detected in the degradation process. The major extent and most rapid rate of dequenching occurred at the earliest reaction times which correlated temporally with the degradation of fibrinogen to a fragment of about 200kD (X) during the first three minutes of the
20 reaction. A second fragment of about 140 kD (Y) first became visible by silver-stained SDS-PAGE after 3 minutes when over 50% of the dequenching had already occurred. Fragment Y reached its maximal concentration between 10 and 30 minutes after digestion was initiated
25 when the dequenching rate had already slowed considerably. The obtainment of the final extent of dequenching was seen at 60 minutes and occurred concomitantly with the presence of the last remaining fragment X in the digestion. A third fragment (D) of
30 about 85 kD appeared at about 10 minutes after digestion was initiated, and remained the prominent fragment from 30 to 300 minutes after the digestion was initiated. A fourth fragment (E) of about 45 kD appeared about 30 minutes after digestion was initiated, and again at 300
35 minutes, but not at 60 minutes and 120 minutes possibly

due to its known, unreliable detection by silver
staining.

During fibrinogenolysis, the total fluorescence
emission at any time is related to the amount of
5   fluorescent-labeled fibrinogen and the amount of
individual fluorescent degradation products in the
sample. The intensity of fluorescence emission F(t) is
described by Equation 1:

$$F(t) = \alpha[fbg(t)] + \Sigma \; \beta_i \cdot [FDP_i(t)]$$

10   where i= X, Y, D, E; and $\alpha= F(0)/[fbg(0)]$ at t=0

The majority of dequenching occurs temporally
during the generation of fragment X. Thus, an initial
reaction rate analysis would relate the rate of
15   dequenching with the first reaction step- the rate of
fragment X generation. All degradation products appear
to have the same fluorescence intensity per molecule, β,
which is justified by the fact that the dequenching
signal reaches steady state long before lysis is
20   complete; i.e., conversion of fragment Y to fragment D
produced little additional dequenching. Plasmin
degradation of one fibrinogen molecule can produce at
least three dequenched fragments that include a single X
fragment and at least two small fragments from the
25   carboxy terminus of the α chains. For the reaction
where plasmin converts one quenched fibrinogen molecule
to n dequenched fragments (of different size, but the
same β) with $n \geq 3$, the Equation 1 becomes either:

Equation 2a $F(t) = \alpha[fbg(t)] + \beta \cdot [n$ dequenched
30   fragment(t)]
          or
Equation 2b $F(t) = \alpha[fbg(t)] + \beta' \cdot [X(t)]$
          where $\beta' = \beta n$ and $\beta' = F_{max}/[fbg(0)]$ as all
fibrinogen -> X

35

Differentiating Eqn. 2(b) where $[fbg(0)] = [fbg(t)] + [X(t)]$, the rate of dequenching is related to X generation by Eqn. 3. One measure of the consistency of this approach is that the expression $[fbg(0)]/(F(0)-F_{max})$

5   which is equal to $1/(\alpha-\beta')$ was measured experimentally and found to remain constant as expected, regardless of reaction conditions.   Equation 3 $1/(\alpha-\beta')$ $dF/dt = d[fbg]/dt = -d[X]/dt$

10   For kinetic analysis where reversible binding and dissociation occurred by distinct binding sites separate from the catalytic site, the rate of complex formation is described by:

Equation 4 $d[plm^b]/dt = k_f[plm^f]\ [\theta_{plm}-plm^b] - k_r[plm^b]$

15   (Diamond et al., 1993, *Biophysical J.* 65:2622-2643) where the total available plasmin binding site concentration, θ_{plm}, is equal to $q \cdot [fbg]$ and $q=2$ initially for two plasmin binding sites per fibrinogen molecule (Lucas et al., 1983, *J. Biol. Chem.* 258:4249-4256).

20   At all times, the free plasmin concentration $[plm^f]$ plus the bound plasmin concentration $[plm^b]$ is equal to the initial plasmin concentration $[plm^o]$. For rapid binding that equilibrates quickly $(d[plm^b]/dt = 0)$, the concentration of bound plasmin is given by (Equation 5):

25   $[plm^b] = \dfrac{(K_d +[plm^o] +q[fbg^o] - \sqrt{(K_d +[plm^o] +q[fbg^o])^2 - 4q[fbg^o][plm^o]}}{2}$

and the initial reaction rate is given as:

Equation 6 $v = k_{cat} \cdot [plm^b]$

30   Using this model, as the initial concentration of fibrinogen increases to very large values (at constant plasmin concentra- tion), the maximum rate goes to $v_{max} = k_{cat} \cdot [plm^o]$. As the initial plasmin concentration increases (at constant fibrinogen), the maximum reaction

35   rate goes to $v_{max} = k_{cat} \cdot q[fbg^o]$. Using 1 nM plasmin and

varying concentrations of fibrinogen from 1.25 to 20 nM
in the method according to the present invention, a
kinetic analysis for the generation of fragment X was
conducted. A reciprocal plot is given for the initial

5   rate of fragment X generation (FIG. 5) calculated from
Equation 3. A linear regression of the experimental
data allowed direct calculation of v_{max} and a
determination of $k_{cat} = 0.479$ sec^{-1}. Using Equations 5 and
6, the K_d of active plasmin binding to fibrinogen was

10 then calculated to be 0.42 μM.
        Although enzyme assays are typically conducted
under conditions of excess substrate, the sensitivity of
the fluorescence-based method of the present invention
allows determination of excess plasmin on nanomolar

15 concentrations of fibrinogen. The fibrinogen substrate
has two binding sites for plasmin, each with fairly high
affinity for plasmin. Thus, it is expected that under
the conditions of excess plasmin, these binding sites
can be saturated with plasmin. This was indeed observed

20 experimentally for the initial stage of the reaction
when the ratio of plasmin to fibrinogen exceeded twice
that of the initial concentration of fibrinogen (FIG.
6). Using values of $k_{cat} = 0.479$ sec^{-1} and $K_d = 0.42$ μM,
the kinetic rates for reactions where the

25 plasmin/fibrinogen ratio was less than one were
accurately predicted with Equations 5 and 6. A large
deviation from the model was observed when the plasmin
was in excess of the initial fibrinogen, a regime where
kinetic formulations typically breakdown. Given the

30 number of lysine binding sites per fibrinogen molecule,
the measured reaction rates reached saturation at the
expected plasmin concentrations, but the value of that
maximum rate was considerably less than predicted. This
may be due to the very short linear regime of the

35 initial rate and the rapid destruction of fibrinogen
such that the assumption of local binding equilibrium

does not hold or the initial concentration of fibrinogen
cannot be used in Equation 5 since it drops so rapidly
at high plasmin concentration.

It was found that preincubation of plasin with ε-
5   amino caproic acid (10 mM) prior to addition of
fluorescent-labeled fibrinogen greatly reduced the
dequenching rate which indicates that plasmin must bind
fibrinogen in order to initiate lysis (FIG. 7).
Addition of excess ε-amino caproic acid, after plasmin-
10 mediated fibrinogenolysis was initiated, resulted in a
dose-dependent inhibition of dequenching (FIG. 8).
Addition of ε-amino caproic acid (10 mM) immediately
inhibited the progression of fibrinogenolysis as
indicated by the cessation of dequenching. This
15 indicated that plasmin can be removed from fibrinogen
either by ε-amino caproic acid disruption of fibrinogen-
plasma complex or by ε-amino caproic acid capture of
desorbing plasmin. Addition of 10 nM plasmin to a
mixture of 10 mM ε-amino caproic acid and 20 nM FITC-
20 fibrinogen resulted in a competition between free
plasmin binding with fibrinogen and plasmin binding with
ε-amino caproic acid. In that competition, some plasmin
was able to bind fibrinogen as indicated by the initial
rate of dequenching.

25   **EXAMPLE 8**
                           Fibrinolysis

An assay for fibrinolysis according to the method
of the present invention was performed in accordance
with materials and methods outlined in Examples 1 and 2.
30 To produce suspensions of fluorescent-labeled fibrin
fibers, thrombin (final concentration of 1 U/ml) was
mixed for 5 seconds with FITC-fibrinogen (80 nM) in
either buffer 1 or buffer 2 after which the
polymerization was monitored until fluorescence
35 quenching was complete and stable for 1 hour. The

daily-fiber suspensions yielded a highly repeatable
extent of quenching and were stable for several hours as
indicated by the stability of the fluorescence signal.
Prior to reactions for fibrinolysis, cuvettes were
5   incubated with 10μM bovine serum albumin for 30 minutes
to minimize adsorption of substrate to the cuvette
walls. Small volumes of the FITC-fibrin were then
pipetted into 2.4 ml of the reaction buffer and
monitored for 200 to 400 seconds to establish the
10   fluorescence baseline before addition of plasmin or
other reagents.

      To illustrate plasmin-mediated fibrinolysis,
plasmin (5 nM) was mixed for about 5 seconds in the
reaction buffer containing between 0.5 to 30 nM FITC-
15   fibrin. Polymerization was monitored by exciting the
reaction mixture with blue light (488 nm) and monitoring
the fluorescence emission at 515 nm using a fluorimeter.
The action of plasmin on FITC-fibrinogen produced a
large increase in fluorescence intensity (dequenching)
20   in a plasmin-dependent manner. During fibrinolysis, the
monomers in the fibrils are degraded with subsequent
loss of fibril/fiber structure. The early dequenching
during fibrinolysis is due to the loss of quenching in
the fibril/fiber due to loss of structure, and the
25   generation of dequenched degradation products. The
initial rate of dequenching was very similar for lysis
of coarse and fine fibers (FIG. 9) and was dependent on
the concentration of fibrin used at constant plasmin
concentration. It was noted that the final dequenched
30   state of lysed fibrin (non-crosslinked) achieved a final
fluorescence emission identical to the final dequenched
state of lysed fibrinogen. Also, the initial rate of
fluorescence change for FITC-fibrin in fibrinolysis
appeared similar to that seen for FITC-fibrinogen in
35   fibrinogenolysis.

EXAMPLE 9

Assays for Inhibitors of Fibrinolysis and Fibrinogenolysis

There are various plasmin inhibitors (also known as
5   "antiplasmins") which may be present in body fluid and
can inhibit fibrinolysis or fibrinogenolysis. Table 2
illustrates several of the physiologically important
plasmin inhibitors.

Table 2

10

plasmin inhibitor	Molecular size (kilodaltons, kD)	Approximate normal plasma/serum conc.
antiplasmin	70 kD	115-150 mg/dl^{-1}
α_1-antitrypsin	51 kD	100-160 mg/dl^{-1}
α_2-macroglobulin	820 kD	130-334 mg/dl^{-1}
C$_1$ inhibitor	90 kD	> 11 mg/dl^{-1}
inter-α-trypsin inhibitor	170 kD	20-70 mg/dl^{-1}
antithrombin III	62 kD	200-400 mg/dl^{-1}

An assay for a plasmin inhibitor's activity in
fibrinolysis or fibrinogenolysis according to the method
of the present invention can be performed in accordance
25 with materials and methods outlined in Examples 1, 2, 7,
and 8.

The sample containing an unknown amount of plasmin
inhibitor activity is added to the reaction buffer
simultaneously with a known amount of plasmin (e.g., 5
30 nM) and FITC-fibrinogen or FITC-fibrin (e.g., 10 nM;
"FITC-substrate"). Lysis of the FITC-substrate is then
monitored by exciting the reaction mixture with blue
light (488 nm) and monitoring the fluorescence emission
at 515 nm using a fluorimeter. A parallel set of
35 standard reactions containing a known amount of the
plasmin inhibitor may be used to determine the amount of

fluorescence emission (dequenching) during the desired
time interval. The extent of interference of lysis of
the FITC-substrate (i.e., prevention of dequenching) at
the end of desired time interval in the reaction
5 containing the sample, is related to the plasmin
inhibitor activity, and hence the plasmin inhibitor
concentration, present in the sample. Thus, from the
percent inhibition of lysis of the FITC-substrate (as
observed by a decrease in dequenching) as compared to
10 the standard(s), can be calculated the plasmin inhibitor
activity (concentration) in the sample. Further, if
desired, a specific antithrombin agent can be added to
prevent thrombin interference.

EXAMPLE 9
Assays for Plasminogen Activators
15

There are various plasminogen activators which may
be present in body fluid and play an indirect role in
fibrinolysis or fibrinogenolysis. Plasminogen is the
inactive precursor of plasmin. Plasminogen activators
20 are proteases that cleave plasminogen into plasmin.
There are at least two biochemically distinct types:
tissue-type (t-PA), approximately 70 kD in size, and
urokinase-type or urinary (u-PA), approximately 33-53
kD. t-PA activating activity is enhanced by fibrin.
25 Other substances, such as streptokinase produced by
certain strains of streptococci, can function to
activate plasminogen. Thus, the method of the present
invention can be used to detect and measure plasminogen
activator activity. Measuring plasminogen activator
30 activity in body fluid is of clinical importance for
several reasons. Plasminogen activators are used in
therapeutic thrombolysis; i.e., therapy designed to
degrade inappropriate fibrin deposition in blood vessels
such as can occur in myocardial infarction. Also, some
35 tumors have been shown to produce uPA, resulting in

plasmin-mediated tumor-associated proteolysis that is
related to the invasive and metastatic potential of
malignant cells in a patient having such a tumor.

9.1 tPA Activity Assay

5   An assay for tPA activity in fibrinolysis or
fibrinogenolysis according to the method of the present
invention can be performed in accordance with materials
and methods outlined in Examples 1, 2, 7, and 8.

A biological sample containing an unknown amount of
10   tPA activity is added to the reaction buffer
simultaneously with a known amount of plasminogen (e.g.,
5 nM) and FITC-fibrin or FITC-fibrinogen (e.g., 10 nM;
"FITC-substrate"). In this instance, it is noted that
FITC-fibrin works better than FITC-fibrinogen because it
15   contains tPA binding site that is not present prior to
polymerization of fibrinogen. Lysis of the FITC-
substrate is then monitored by exciting the reaction
mixture with blue light (488 nm) and monitoring the
fluorescence emission at 515 nm using a fluorimeter. A
20   parallel set of standard reactions containing a known
amount of tPA may be used to determine the amount of
fluorescence emission (dequenching) during the desired
time interval (i.e., in calibrating the assay). The
extent of lysis of the FITC-substrate (i.e.,
25   dequenching) at the end of desired time interval in the
reaction containing the sample, is related to the tPA
activity, and hence the tPA concentration, present in
the sample. Thus, the amount of lysis of the FITC-
substrate (as observed by the amount of dequenching) as
30   compared to the standard(s), can be calculated the tPA
activity (concentration) in the sample. Further, if
desired, to increase the sensitivity, tPA may be pre-
incubated with plasminogen in the presence of unlabeled
fibrin for a fixed period of time (e.g., 15 to 90
35   minutes) before addition of FITC-substrate. The

detection limit is at least 20 picomolar tPA (single chain, recombinant) under routine conditions.

9.2   uPA Activity Assay

An assay for uPA activity in fibrinolysis or
5   fibrinogenolysis according to the method of the present invention can be performed in accordance with materials and methods outlined in Examples 1, 2, 7, and 8.

A biological sample containing an unknown amount of uPA activity is added to the reaction buffer
10   simultaneously with a known amount of plasminogen (e.g., 5 nM) and FITC-fibrin or FITC-fibrinogen (e.g., 10 nM; "FITC-substrate"). Lysis of the FITC-substrate is then monitored by exciting the reaction mixture with blue light (488 nm) and monitoring the fluorescence emission
15   at 515 nm using a fluorimeter. A parallel set of standard reactions containing a known amount of uPA may be used to determine the amount of fluorescence emission (dequenching) during the desired time interval (i.e., in calibrating the assay). The extent of lysis of the FITC-
20   substrate (i.e., dequenching) at the end of desired time interval in the reaction containing the sample, is related to the uPA activity, and hence the uPA concentration, present in the sample. Thus, the amount of lysis of the FITC-substrate (as observed by the
25   amount of dequenching) as compared to the standard(s), can be calculated the uPA activity (concentration) in the sample. To increase the sensitivity, uPA may be preincubated with plasminogen in the presence of unlabeled fibrin for a fixed period of time (e.g., 15 to
30   90 minutes) before addition of FITC-substrate. The detection limit is at least 200 picomolar uPA under routine conditions.

EXAMPLE 10

35   In another embodiment, using the method of the present invention, and in accordance with materials and

methods outlined in Examples 1-9, naturally-occurring
molecular abnormalities fibrinogen ("dysfibrinogens")
can be detected clinically. A sample suspected of
containing a dysfibrinogen can be isolated from an
5   individual. The apparent dysfibrinogen can be
fluorescently labeled, and used as a fluorescent-labeled
substrate in one or more of the assays measuring enzyme
activity. For example, a dysfibrinogen has been
described wherein there is a mutation in the thrombin-
10   cleavage site (position 16 of the Aα chain) involved in
fibrinogen polymerization to fibrin. Therefore, in an
assay including the sample and a known amount of
thrombin, interference with quenching as compared to a
standard reaction containing a known amount of
15   fibrinogen may be used to determine if the suspected
dysfibrinogen is present in the sample.

EXAMPLE 11

While the preceding Examples disclose embodiments
20   that illustrate the fluorescence-based method of the
present invention in detecting and measuring activity of
components involved or active in clot formation or
dissolution, it will be apparent to those skilled in the
art that the fluorescence-based method of the present
25   invention can equally be applied to the detection and
measurement of components active in other processes,
provided that critical to such a process there is a
protein substrate that is a template with which
molecular assemblies that occur in the body are
30   recreated. More particularly, the substrate is a dimeric
or oligomeric molecule, preferably naturally occurring
as a body fluid component, which a) can be labeled with
fluorescent molecules; b) is modifiable in either an
enzyme-catalyzed polymerization reaction or an enzyme-
35   catalyzed degradation or dissolution reaction; c) where
upon modification of the fluorescent-labeled substrate,

- 35 -

a change in fluorescence emission results; and d)
measurement of the difference between the fluorescence
emission as a result of the reaction and the basal level
of fluorescence emission (prior to substrate
5   modification) directly relates to substrate
concentration and/or enzyme activity and indirectly
relates to the presence of absence of other components
involved in that process.

In one embodiment, an assay for proteolytic
10 activity of a sample containing an unknown amount of a
proteolytic enzyme using the method of the present
invention can be performed in accordance with materials
and methods outlined in Examples 1, 2, 7, and 8. FITC-
fibrin or FITC-fibrinogen can be used as a substrate to
15 be degraded in detecting and measuring a sample
containing an unknown amount of activity of a specific
protease, including such proteases as trypsin, elastase,
and papain. The extent of lysis of the FITC-substrate
(i.e., dequenching) at the end of desired time interval
20 in the reaction containing the sample, is related to the
protease activity, and hence the protease concentration,
present in the sample. Standard reactions containing a
known amount of the protease may be run in parallel to
calibrate the assay.

25   In another embodiment of the method of the present
invention, and according to methods and materials
outlined in Examples 1-9, a process or pathway other
than clot formation or dissolution, and the components
involved therein, can be detected and measured. For
30 example, procollagen may serve as the substrate which is
fluorescently labeled. Fluorescent-labeled procollagen
is a substrate which can be added as a monomer or in a
polymerized state in a reaction mixture along with a
sample containing an unknown amount of activity of a
35 component such as collagenase, gelatinase, and/or
collagenase inhibitor to detect and measure the

component in that sample using the method according to
the present invention.

      Although the present invention has been
described in some detail by way of illustration and
5 example for purposes of clarity and understanding,
various modifications will become apparent to those
skilled in the related arts from the foregoing
description and figures. Such modifications are
intended to be included within the spirit of this
10 application and within the scope of the appended claims.

What is claimed is:

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1.   A method for measuring in a sample a component involved in a pathway which leads to polymerization of a substrate, wherein the component is selected from the group consisting of an enzyme, enzyme inhibitor, and
5   enzyme activator, said method comprises:
        (a) contacting the sample in a reaction mixture with the substrate which has been labeled with fluorescent molecules, wherein the substrate is a dimeric or oligomeric protein modifiable in an enzyme-
10   catalyzed polymerization reaction and is a template on which molecular assemblies occur;
        (b) incubating the reaction mixture containing the sample and fluorescent-labeled substrate for a sufficient time to allow for a reaction to occur in
15   solution, and exposing the reaction mixture to an excitation wavelength specific for the fluorescent label; and
        (c) detecting the fluorescence emission of the reaction wherein reduced fluorescence emission or
20   quenching caused by enhanced neighbor-neighbor interactions between fluorescent label molecules in a substrate upon polymerization, relative to a basal level of fluorescence, is indicative of substrate polymerization and may be used as a measure of the
25   component's activity in the sample.

    2.   The method according to claim 1, wherein the sample is a body fluid.

30   3.   The method according to claim 2, wherein the body fluid is a fluid selected from the group consisting of blood, serum, and plasma.

    4.   The method according to claim 1, wherein the enzyme
35   inhibitor is a pharmaceutical.

5.   The method according to claim 1, wherein the enzyme activator is a pharmaceutical.

6.   The method according to claim 1, wherein the
5 component in the sample to be measured is a component selected from the group consisting of an enzyme inhibitor and an enzyme activator; the reaction mixture with which the sample is contacted contains the fluorescent-labeled substrate, and further contains an
10 enzyme which catalyzes polymerization of the substrate and to which the component's activity is directed; and the basal level of fluorescence is that measured from a reaction containing the enzyme and substrate in a reaction mixture.

15
7.   The method according to claim 1, wherein the pathway is fibrinogen polymerization into fibrin, wherein the substrate is fibrinogen, and wherein the enzyme to be measured in the sample is thrombin.

20
8.   The method according to claim 6, wherein the component is an enzyme inhibitor selected from the group consisting of heparin and antithrombin, wherein the substrate is fluorescent-labeled fibrinogen, and wherein
25 the enzyme is thrombin.

9.   The method according to claim 1, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

30 10.   The method according to claim 6, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

11.   The method according to claim 8, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

35

12.   A method for measuring in a sample a component involved in a pathway which leads to degradation or dissolution of a substrate, wherein the component is selected from the group consisting of an enzyme, enzyme
5 inhibitor, and enzyme activator, said method comprises:
     (a) contacting the sample in a reaction mixture with the substrate which has been labeled with fluorescent molecules, wherein the substrate is a dimeric or oligomeric protein modifiable in an enzyme-
10 catalyzed degradation or dissolution reaction and is a template on which molecular assemblies occur;
     (b) incubating the reaction mixture containing the sample and fluorescent-labeled substrate for a sufficient time to allow for a reaction to occur in
15 solution, and exposing the reaction mixture to an excitation wavelength specific for the fluorescent label; and
     (c) detecting the fluorescence emission of the reaction wherein increased fluorescence emission or
20 dequenching caused by an increase in the distance between neighboring molecules of fluorescent label as a result or unfolding or cleavage of the substrate, relative to a basal level of fluorescence, is indicative of substrate degradation or dissolution and
25 may be used as a measure of the component's activity in the sample.

     13.   The method according to claim 12, wherein the sample is a body fluid.

30
     14.   The method according to claim 13, wherein the body fluid is a fluid selected from the group consisting of blood, serum, and plasma.

35   15.   The method according to claim 12, wherein the enzyme inhibitor is a pharmaceutical.

16. The method according to claim 12, wherein the enzyme activator is a pharmaceutical.

17. The method according to claim 12, wherein the component in the sample to be measured is a component selected from the group consisting of an enzyme inhibitor and an enzyme activator, and the reaction mixture with which the sample is contacted contains the fluorescent-labeled substrate, and further contains an enzyme which catalyzes degradation or dissolution of the substrate and to which the component's activity is directed.

18. The method according to claim 12, wherein the pathway is a process selected from the group consisting of fibrinogenolysis and fibrinolysis, wherein the substrate is fibrinogen when the pathway is fibrinogenolysis and wherein the substrate is fibrin when the pathway is fibrinolysis, and wherein the enzyme to be measured in the sample is plasmin.

19. The method according to claim 17, wherein the component is an enzyme inhibitor selected from the group consisting of antiplasmin, α_1-antitrypsin, α_2-macroglobulin, C_1 inhibitor, inter-α-trypsin inhibitor, and antithrombin III; wherein the substrate is fluorescent-labeled fibrinogen or fluorescent-labeled fibrin, and wherein the enzyme is plasmin.

20. The method according to claim 17, wherein the component is a plasminogen activator; wherein the substrate is fluorescent-labeled fibrinogen or fluorescent-labeled fibrin, and wherein the enzyme is plasminogen.

21. The method according to claim 20, wherein the plasminogen activator is selected from the group consisting of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).

5

22. The method according to claim 12, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

23. The method according to claim 17, wherein the
10   fluorescent label is fluorescein isothiocyanate (FITC).

24. The method according to claim 19, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

15   25. The method according to claim 20, wherein the fluorescent label is fluorescein isothiocyanate (FITC).

26. A method for detecting a dysfibrinogen in a sample, said method comprises:
          (a) labeling the dysfibrinogen with fluorescent
20   molecules;
          (b) incubating a reaction mixture containing the fluorescent-labeled dysfibrinogen and an enzyme having activity against fibrinogen for a sufficient time to
25   allow for a reaction to occur in solution, and exposing the reaction mixture to an excitation wavelength specific for the fluorescent label; and
          (c) detecting the fluorescence emission of the reaction wherein a change in fluorescence emission,
30   relative to a basal level of fluorescence determined from reaction containing fibrinogen and the enzyme, is indicative of the dysfibrinogen's activity.
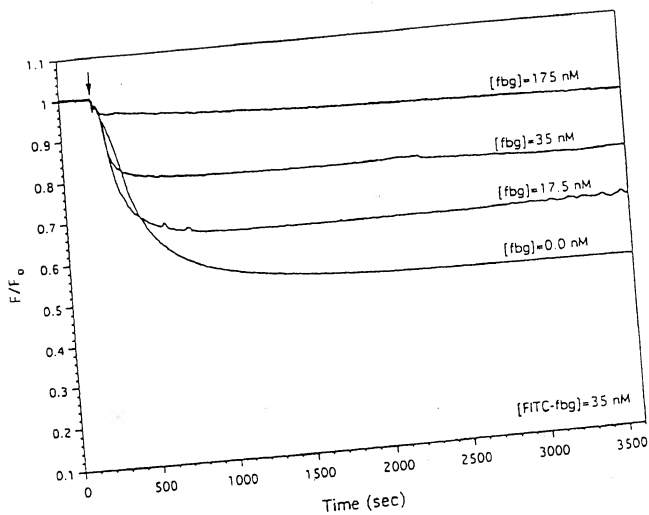
35

1/11
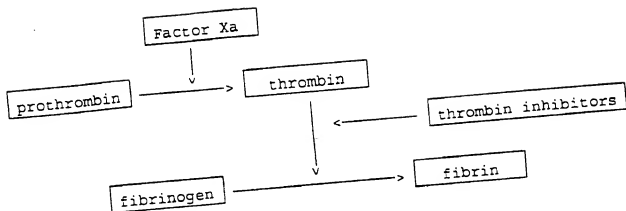
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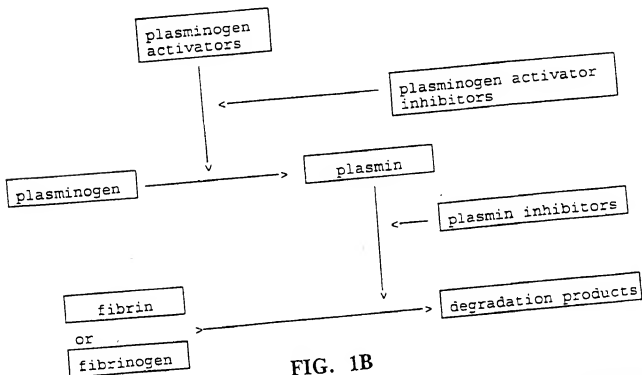
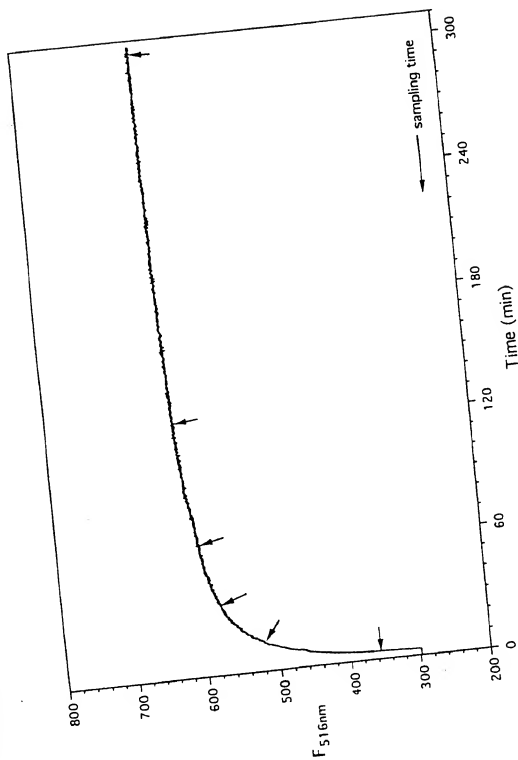
FIG. 1B

2/11
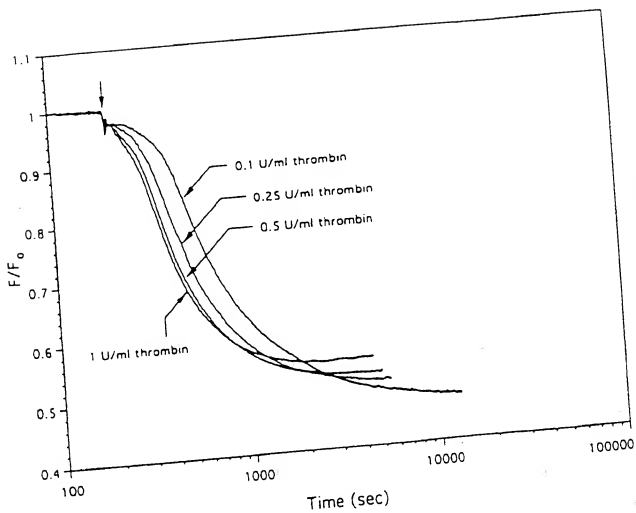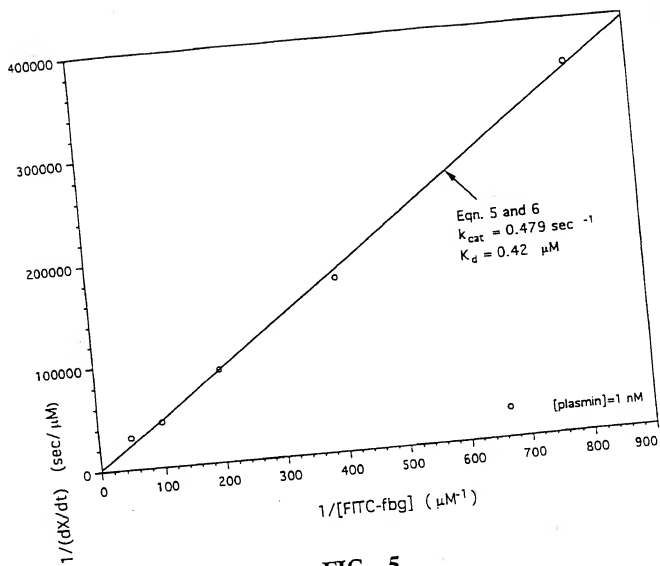

FIG. 2A

3/11
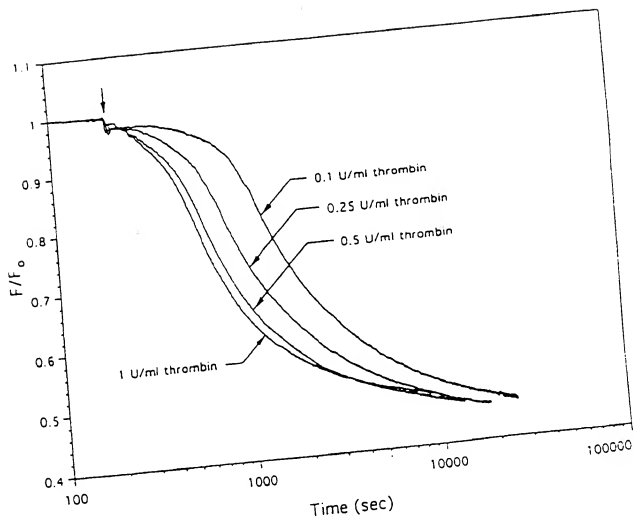
FIG. 2B

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FIG. 3A

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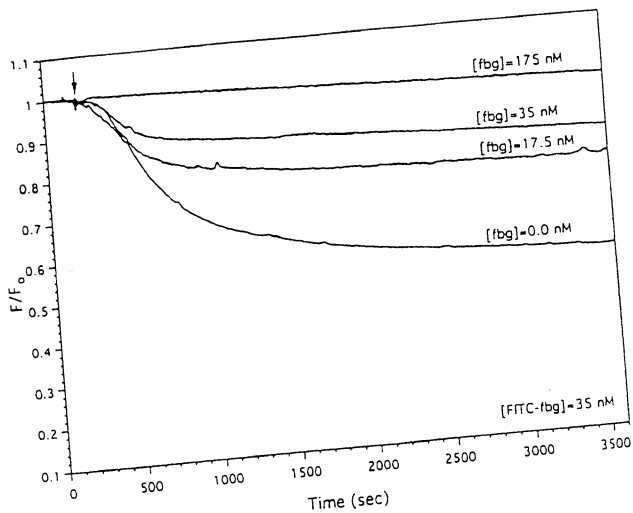
FIG. 3B

FIG. 4

FIG. 5

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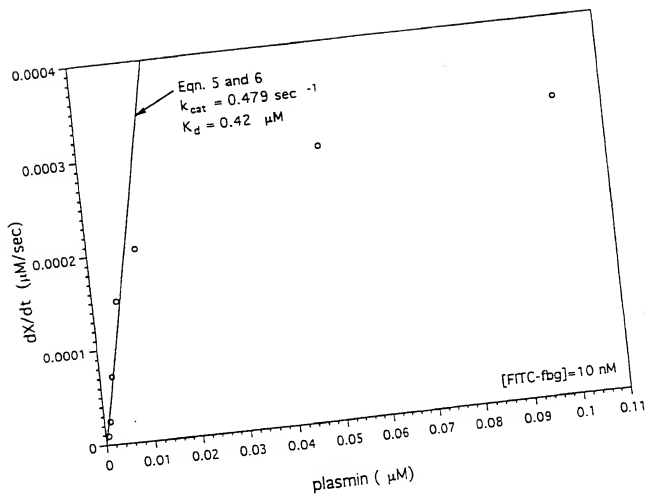
FIG. 6

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FIG. 7

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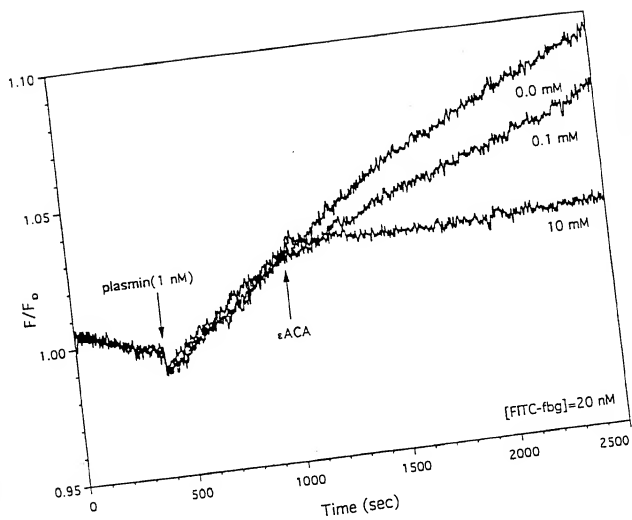
FIG. 8

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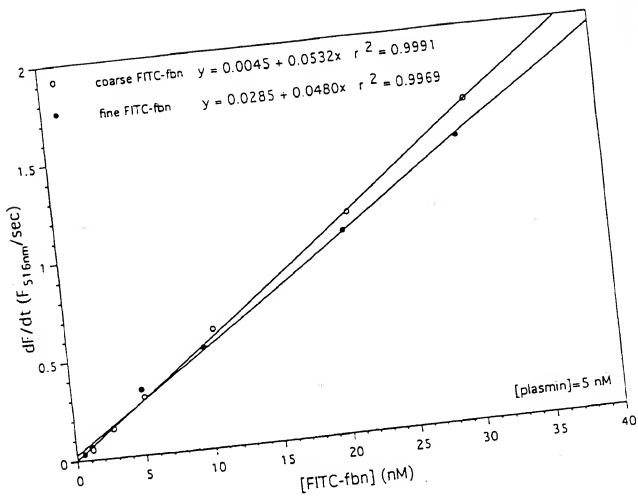
FIG. 9

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,668,623 (KINNUNEN ET AL.) 26 May 1987, column 4, lines 44-64; column 5, lines 4-12; column 7, line 7.	12-14
A	US, A, 4,046,635 (MOROZ) 06 September 1977, see entire document	1-26
A	US, A, 5,071,745 (TRISCOTT ET AL.) 10 December 1991, see entire document.	1-26

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

01 APRIL 1996

Date of mailing of the international search report

09 APR 1996

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Washington, D.C. 20231

Facsimile No.   (703) 305-3230

Authorized officer

CAROL A. SPIEGEL

Telephone No.   (703) 308-0196

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12Q 1/37, 1/56; G01N 33/533

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/4, 13, 23, 24; 436/69, 536, 546

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/4, 13, 23, 24, 968; 436/69, 536, 546, 800

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG
search terms: fluoresc?, fibrin, fibrinogen, collagen, procollagen, polymeriz?, degrad?, substrate